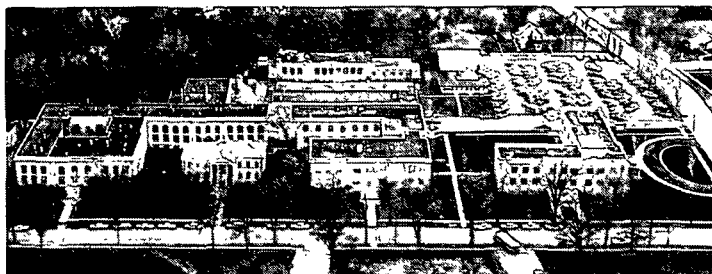


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**STUDY OF THE REGENERATING CELL WALLS OF DOUGLAS-FIR PROTOPLASTS.  
II. CELL WALL GLYCOPROTEIN CHARACTERISTICS**

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Study of the Regenerating Cell Walls of Douglas-Fir Protoplasts  
II. Cell Wall Glycoprotein Characterization

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Abstract. A glycoprotein was isolated from the regenerating cell walls of Douglas-fir protoplasts by two different techniques and partially characterized. One extraction method using sodium borohydride effectively removed protein from the wall material and stabilized the polysaccharides against hydrolysis while severely degrading the protein. A second and milder extraction method conducted at lowered temperatures with a mild alkaline solution removed limited amounts of wall glycoprotein but with little degradation. The glycoprotein mainly contains glycine, alanine, glutamic acid, serine, glucose, galactose, and mannose, yielding a single peak in the ultracentrifuge with an estimated molecular weight of 282,000 but resolvable by isoelectric focusing into two species with isoelectric points of 4.2 and 4.4. Although hydroxyproline can be found in the cell wall hydrolyzates of suspension cells, the regenerating wall on protoplasts lacks this amino acid.

Introduction

Over the past 20 years several investigators have confirmed the presence of proteins in the cell walls of higher plants, ending a debate which lasted almost a century (Lampert, 1965). Two types of glycoprotein have been found in cell walls. Those abundant in the rare amino acid, hydroxyproline, were named extensin (Lampert, 1963) and have been investigated extensively in carrot (Sadava and Chrispeels, 1978), excised *Avena* coleoptiles (Cleland, 1968; Fujii, 1978), and sycamore suspension (Lampert, 1978) systems. The other glycoprotein was reported to be hydroxyproline-poor (Brown and Kimmins, 1979a) and synthesized largely in response to mechanical wounding. It was a high molecular weight

substance rich in alanine, glycine and glutamic acid (Brown and Kimmins, 1978) and deposited in the cell walls of bean leaves and other intact leaf tissues after multiple surface lesions were made.

It was considered that cell wall regeneration on plant protoplasts might provide a unique opportunity to explain some fundamental aspects of cell wall biogenesis (Willison, 1976); especially with regard to the role of wall proteins (Lampert, 1978). In the present study the protein deposited in the regenerating cell walls of Douglas-fir protoplasts was partially characterized and then compared with the two types of cell wall glycoprotein described above. The protein found in the cell walls of Douglas-fir suspension cells from which the protoplasts were generated was examined for comparative purposes.

#### Materials and Methods

Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] regenerating cell walls were isolated from both needle- and hypocotyl-derived protoplasts originating from suspension cultures as already described (Robinson and Johnson, 198 ). The protoplast medium was enriched with  $^{14}\text{C}$ -L-proline and factors necessary for proline hydroxylation (Sadava and Chrispéels, 1971). The recovered cell walls represented growth from 0 to 6 days after the cell wall degrading enzymes were removed.

#### Protein Extractions

Two methods of alkali extraction were used separately to remove and isolate the cell wall-bound protein material. The first approach was purported to limit the degradation of carbohydrate through  $\text{NaBH}_4$  reduction. The protein extracted is called the wall elimination produce (WEP) (Lampert, 1979). The second technique involved a milder alkali extraction (MAE) aimed at limited degradation of nascent protein (Brown, 1979).

# 1. Wall Elimination Product (WEP)

The WEP preparation was made by mixing a 1 g composite of cell walls (representing 0 to 6 days cell wall regeneration) at 45°C with 50 mL DMSO, 40 mL of 25% ethanol, and 10 mL of 2N NaOH in which 3.8 g of NaBH<sub>4</sub> was dissolved. After 5 h the mixture was filtered through a coarse sintered glass funnel, the filtrate discarded, and the cake washed with 100 mL of 95% ethanol. The air-dried cake was then washed with 150 mL of distilled water. The resulting filtrate was neutralized with acetic acid, evaporated at 40°C to 50 mL, mixed with 200 mL of 95% ethanol, and left standing over night in a graduated cylinder. The supernatant was drawn off leaving a precipitate. The precipitate was further isolated by centrifugation and then allowed to air dry.

The ethanol-precipitated WEP was fractionated by gel permeation chromatography. A Sephadex G-75 column (1.2 x 35 cm) with a 150 mm hydrostatic head, 6.3 mL/h flow rate, and a void volume of 11.7 mL was used. The excluded volume was determined with equine ferritin. WEP (2 mg/mL) was added and eluted at room temperature in water. Column elution was followed at 280 nm with ISCO models 659 scanner and UA-5 absorbance monitor. Twenty-minute fractions were collected and freeze-dried.

Several fractions were chosen for further analysis based upon their position on the chromatogram (see Fig. 1). Fraction numbers 7, 12, 22, and 28 were separately acid hydrolyzed as described earlier (Robinson, and Johnson, 198.) for amino acid analysis. Radioactive fractions collected from the amino acid analyzer were counted in a scintillation counter.

Other fractions (numbers 10, 14, 18, and 23) were isoelectric focused to establish the character of the WEP extracted protein. An LKB 2117 Multiphor

electrofocusing apparatus at a constant power of 25 W was employed using LKB PAGplate gels having an ampholyte range of pH 3.5 to 9.5. Parallel samples of each fraction were run to determine if the WEP was a glycoprotein. After focusing duplicate samples on the gel, they were separated and stained with either Coomassie Blue or periodic acid-Schiff's reagent (Kipatany and Zebrowski, 1973) for the respective detection of protein or carbohydrate.

## 2. Mild Alkali Extraction (MAE)

The second technique patterned after Brown and Kimmins (1978) was used to remove cell wall-bound proteins with only moderate degradation. Varying amounts of freeze-dried regenerating cell walls (from 25 to 300 mg) from cell cultures of 0 to 6 days regeneration were mixed with 10 mL of 1N NaOH and carefully maintained at 2°C with occasional stirring. The solution was neutralized after 30 min with glacial acetic acid. The cell wall residue was sedimented and the supernatant collected and dialyzed against distilled water. The dialysis was performed over 24 h with 3 exchanges of water to remove contaminating salts or low molecular weight carbohydrates. The dialyzate was freeze-dried after mixing it with pyridine to make a 10% solution.

After dissolution in 0.02M NaOH-glycine buffer at pH 9.0, the cell wall extract was eluted with the same buffer from a Sephadex G-200 column (2.5 x 50 cm) with a 150 mm hydrostatic head and 9.2 mL/h flow rate or from a G-75 column described above. The void volume was determined with Blue Dextran 2000. The MAE protein was found to be totally excluded by the G-200 column and was collected, dialyzed, and freeze-dried as before. Samples from 2 to 6 days cell wall regeneration were separately evaluated for their isoelectric points and homogeneity. Samples from days 0 through 6 were analyzed for their carbohydrate and amino acid content as described earlier (Robinson and Johnson, 198 ).

Molecular weight of a sample from day 6 was estimated by employing a Beckman Model E analytical ultracentrifuge for determinations of the sedimentation and diffusion constants.

#### <sup>14</sup>C-Proline in Cell Walls of Douglas-Fir Cultured Cells

To determine if a protein rich in proline or hydroxyproline was deposited in the regenerating cell wall, <sup>14</sup>C-proline was added to the medium at a level of 0.1 µCi/mL at zero regeneration time. After 6 days the cell walls were assessed for the source of their radioactivity after acid hydrolysis. Fractions were collected from an amino acid analyzer for scintillation counting. Cell suspensions cultured in both the suspension and protoplast media (Robinson and Johnson, 198 ) were similarly given <sup>14</sup>C-proline and the amino acid composition of cell walls analyzed after 6 days. Appropriate fractions were collected for scintillation counting of the proline and hydroxyproline peaks.

#### Results

Two extraction schemes used on regenerating cell wall samples provided insight into the nature of the deposited protein. For clarity the analytical results for both extracts are presented concurrently.

Chromatography of the two cell wall extracts produced quite different results. A chromatogram of the WEP preparation from a G-75 column is shown in Figure 1 with the void volume marked by the exclusion of ferritin. The most striking feature of Figure 1 is the demonstration of extract heterogeneity. The MAE yielded much less material for chromatography since only nascent glycoproteins are expected to be removed under the mild extraction conditions (Brown, 1979). Chromatography on a G-200 column resulted in only an excluded fraction. Due to the faster flow rate, a G-75 column was used in subsequent runs and the excluded fraction similarly collected.

Isoelectric focusing of proteins from both extraction procedures provided valuable data on the cell wall regeneration and extraction processes. Four WEP fractions indicated in Figure 1 were isoelectric focused as shown in Figure 2. All samples were readily soluble and migrated to isoelectric pH's between 3.0 and 5.0. Figure 2 shows that sample heterogeneity increases with decreasing molecular weight. It is also important to note that the centroid of the focused fractions is about 4.3. In addition, equivalent bands were observed to react with both carbohydrate and protein stains demonstrating that the WEP fragments were glycoprotein.

The MAE protein was also electrofocused to determine sample homogeneity as a function of cell wall regeneration time. Figure 3 shows the isoelectric positions of cell wall extracts from day 2 through 6. The samples, although partially soluble, were all resolved into bands at pH 4.2 and 4.4 and sedimented as a homogeneous material. Although the MAE glycoprotein was not homogeneous by isoelectric focusing (Fig. 3), it behaved as a single peak in the ultracentrifuge. Therefore, from determined S and D values and an assumed partial specific volume of 0.74 (Smith, 1970), the molecular weight of the two components sedimenting as one was estimated at 282,000.

Amino acid compositions from the acid hydrolyzates of proteins from both MAE and WEP extractions are displayed in Table 1. MAE proteins as a function of regeneration time and WEP proteins of fractions from Figure 1 are compared with a wound-induced cell wall glycoprotein of Phaseolus vulgaris (Brown and Kimmins, 1978).

The carbohydrate portion of the MAE glycoprotein is shown in Figure 4. The dominant sugar was glucose. The data in this determination suffered only in an absolute sense from the handling and weighing of minute amounts (200 to 630  $\mu$ g) of sample although the general relationship can be appreciated.

Radioactive proline was fed to suspension cells and protoplasts under various conditions subjecting the cells to increasing osmotic and mechanical stress. Cell wall acid hydrolyzates were made and only the proline and hydroxyproline peaks were collected from the amino acid analyzer for counting in a scintillation counter. The results are displayed in Table 2 and demonstrate that as the medium molarity of the suspension cultures was increased a reversal in the ratios of hydroxyproline to proline was observed. Protoplasts which were subjected to both the high molarity conditions and the enzymatic removal of the cell wall exclusively yielded the radioisotope as proline.

### Discussion

In the past 15 years the major obstacle in the characterization of cell wall proteins has been extraction of the material intact. Since the proteins extensively studied to date have been glycoproteins, additional linkages obviously compound the problem. Of the two extraction approaches used in this study, the WEP procedure appears to degrade the protein structure while the MAE removes the newly deposited and possibly less highly glycosylated or cross-linked protein (Brown, 1979). Figures 1 and 2 show the extent of the WEP degradation. Over 95% of the material is included on G-75. In contrast, the MAE protein only exhibits marginal solubility in water suggesting that it is less highly glycosylated than the WEP. Less overall degradation of the protein appears to occur as indicated by its high apparent molecular weight.

Isoelectric focusing experiments revealed that the WEP fragments focused in a pH range centered about pH 4.3 which is similar to the less degraded protein of the MAE. The high resolving power of isoelectric focusing taken together with the single peak seen in the ultracentrifuge indicate that the two components of the MAE seen in Figure 3 must be of very similar size.



Amino acid analyses of the two wall extracts differed significantly in their content of hydroxyproline. The more severe conditions of the WEP procedure probably resulted in a contribution from residual suspension cell walls remaining after protoplast formation. Since the immediate precursor of hydroxyproline is proline (Lamport, 1963), the results in Table 2 confirm the absence of hydroxyproline synthesis under the conditions and during the period of wall regeneration studied. This finding suggests that extensin was not deposited during wall regeneration in this investigation. Other evidence to support this view is seen in the vastly different amino acid analysis (Lamport, 1969), carbohydrate content (Miller et al., 1972), and isoelectric point (Brysk and Chrispeels, 1972) of extensin compared with the glycoprotein extracted in this present study. This result is perhaps surprising since the synthesis of extensin has been related to mechanical (Sadava and Chrispeels, 1978) or fungal (Esquerré-Tugayé et al., 1979) wounding although it is also apparently related to the state of cellular development (Steward et al., 1974; Roberts and Northcote, 1972).

Another cell wall protein gaining prominence in recent years is a hydroxyproline-poor wound-induced glycoprotein (Brown and Kimmins, 1979a). When compared with the MAE glycoprotein extracted from the regenerating cell walls of Douglas-fir protoplasts, there appear to be significant similarities. The amino acid composition of the wall protein of Phaseolus vulgaris (Brown and Kimmins, 1978) was compared with the similarly extracted material (see columns 2 through 6, Table 1) from the regenerating wall. In general, the amino acid compositions are ordered in parallel within experimental error although subtle differences do exist. The carbohydrate portions of the bean and Douglas-fir glycoproteins have all of the same sugars (Brown et al., 1975) although Brown and Kimmins (1978) found that the absolute quantities were quite variable. The bean glycoprotein exhibited an isoelectric point of 3.63 (Brown and Kimmins, 1979a) and

a molecular weight of 520,000 (Brown and Kimmins, 1979b). While the bean glycoprotein isoelectric point differed from that deposited in the regenerating Douglas-fir cell wall, the variance may be an acceptable species difference. The bean glycoprotein molecular weight is a little less than twice the 282,000 reported in the present study making it tempting, but for the assumed partial specific volume, to speculate that the bands in Figure 3 are two nearly equal parts of one original glycoprotein. In addition a final similarity evidenced is that both the bean and Douglas-fir glycoprotein appear to be synthesized and deposited in response to wounding or stress and neither was catabolized over the period studied.

Brown and Kimmins have not speculated on the exact role which their wound-induced cell wall glycoprotein appears to play. Both their mechanical disruption of the cell wall and the enzymatic attack used to generate protoplasts in this study resulted in the deposition of a protein. The protein's rapid synthesis upon wounding in both bean and Douglas-fir strongly suggests a role in extracellular repair.

The presence of a cell wall wound-induced glycoprotein rich in glycine, alanine, and glutamic acid has been further demonstrated in fava beans, corn, tobacco, and rhubarb (Brown, 1979). The extensin-related work, however, has emphasized hydroxyproline and a few associated amino acids (e.g.: serine, lysine, valine, tyrosine, and tryptophan) (Lamport, 1979) and ignored the presence of other cell wall amino acids which were not part of the model (Lamport, 1970, 1965). In green algae (Thompson and Preston, 1967) and in both woody gymnosperms (Scurfield and Nicholls, 1976) and angiosperms (Lüdke and Liefländer, 1970), cell wall proteins rich in many of the wound-generated amino acids in Table 1 have been found, suggesting that the occurrence of this protein may be widespread throughout the plant kingdom.

Portions of this work were used by one of the authors (KWR) as partial fulfillment of the requirements for the Ph.D. degree at The Institute of Paper Chemistry.

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Table 1. The molar percentages of amino acids in acid hydrolyzed cell wall glycoproteins extracted from the regenerating cell wall compared with a wound-induced glycoprotein from bean cell walls.

Amino Acid <sup>a</sup>	Wounded Bean Cell Walls <sup>b</sup>	MOLAR PERCENTAGES									
		Douglas-fir Regenerated Cell Walls									
		Regeneration Time <sup>c</sup> , days					G-75 Fraction No. <sup>d</sup>				
		0	1	2	4	6	7	12	22	28	
Glycine	11	15	18	20	16	14	5	6	5	11	
Alanine	11	16	13	9	16	13	7	7	7	7	
Aspartic Acid	7	11	11	10	13	12	12	13	15	13	
Serine	7	10	11	13	9	8	5	4	4	11	
Glutamic acid	10	6	7	8	11	9	13	15	13	20	
Leucine	7	7	8	8	6	7	10	10	10	5	
Threonine	5	7	5	5	6	7	4	4	4	3	
Lysine	7	6	5	6	6	6	5	4	3	6	
Proline	7	5	3	2	4	5	5	4	6	2	
Arginine	4	4	4	4	3	4	6	5	4	2	
Valine	6	4	3	3	2	5	7	7	6	0	
Phenylalanine	4	3	3	4	2	3	7	6	5	1	
Isoleucine	5	2	2	3	2	3	6	5	4	2	
Tyrosine	4	2	3	3	2	2	4	3	7	0	
Histidine	1	2	2	2	1	1	2	2	2	6	
Methionine	1	4	4	4	3	4	0	1	1	0	
Hydroxyproline	2	0	trace	0	trace	trace	1	2	3	0	

<sup>a</sup>Neither cysteine nor cystine were detected.

<sup>b</sup>Brown and Kimmins (1978).

<sup>c</sup>MAE proteins.

<sup>d</sup>WEP proteins.

Table 2 The recovery of  $^{14}\text{C}$ -proline from the cell walls of suspension cells and protoplasts after 6 days uptake. Increasing the osmotic stress in suspension cultures results in a decrease of isotope incorporation as hydroxyproline. The high molarity medium and enzymatic removal of the cell wall results in the exclusive appearance of proline.

Cell Type and Condition	% Relative Radioactivity	
	Proline	Hydroxyproline
Suspension cells (Normal medium)	43	57
Suspension cells (High molarity medium)	65	35
Protoplast regenerated wall	100	0

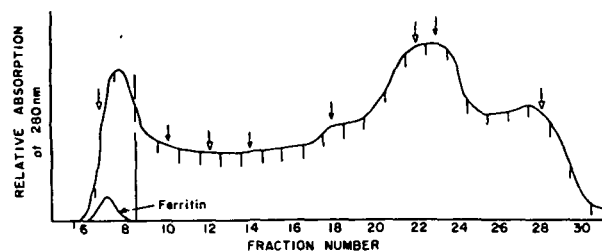


Fig. 1 G-75 chromatogram of the WEP extract from a regenerating cell wall composite as it absorbed at 280 nm. The (✓) positions represent the fractions used for amino acid analysis (Table 1) while the (↓) represent those used in isoelectric focusing (Fig. 2).

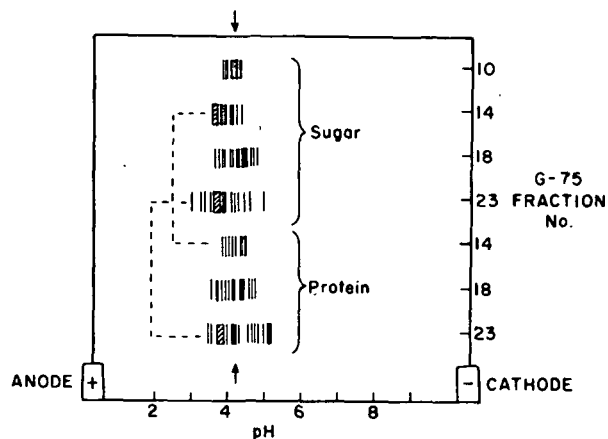


Fig. 2 Electrofocused pattern of WEP extracted cell wall protein. The four WEP fractions are shown to be heterogeneous and centered about pH 4.3 (see arrow). The complementary carbohydrate and protein staining indicates that the wall material extracted was a glycoprotein.

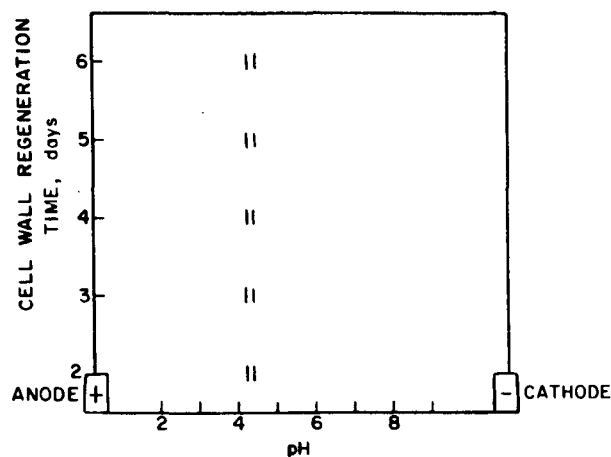


Fig. 3 Isoelectric focusing of MAE. The extract isoelectric focused in two bands from 2 to 6 days regeneration time suggesting that no more than two proteins are deposited over that period.

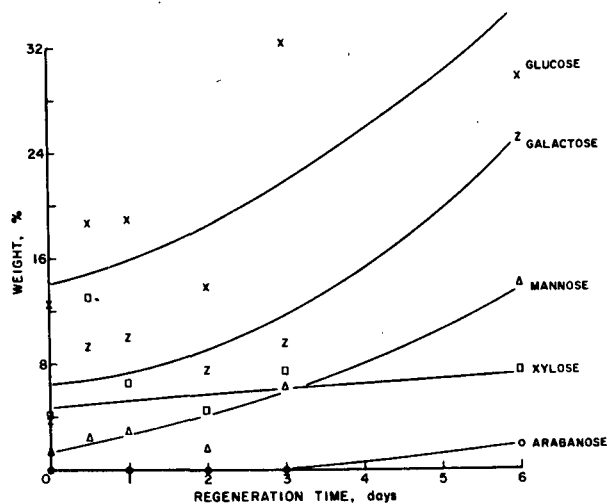


Fig. 4 Carbohydrate analysis of the MAE glycoprotein over 6 days wall regeneration time. Glucose is the dominant sugar, although parallel increases over the period studied are seen with galactose and mannose. These increases suggest additional protein glycosylation with time.